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**Protein kinase C gamma interneurons in the rat medullary dorsal horn: distribution and synaptic inputs of these neurons, and subcellular localization of the enzyme**

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**Running head:** PKC $\gamma$  interneurons in rat MDH

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## ABSTRACT

The  $\gamma$  isoform of protein kinase C (PKC $\gamma$ ), which is concentrated in interneurons in the inner part of lamina II (II<sub>i</sub>) of the dorsal horn, has been implicated in the expression of tactile allodynia. Lamina II<sub>i</sub> PKC $\gamma$  interneurons were shown to be activated by tactile inputs and to participate in local circuits through which these inputs can reach lamina I, nociceptive output neurons. That such local circuits are gated by glycinergic inhibition and that A- and C-fibers low threshold mechanoreceptors (LTMRs) terminate in lamina II<sub>i</sub> raise the general issue of synaptic inputs to lamina II<sub>i</sub> PKC $\gamma$  interneurons. Combining light and electron microscopic immunochemistry in the rat spinal trigeminal nucleus, we show that PKC $\gamma$ -immunoreactivity is mostly restricted to interneurons in lamina II<sub>i</sub> of the medullary dorsal horn, where they constitute 1/3 of total neurons. The majority of synapses on PKC $\gamma$ -immunoreactive interneurons are asymmetric (likely excitatory). PKC $\gamma$ -immunoreactive interneurons appear to receive exclusively myelinated primary afferents in type II synaptic glomeruli. Neither large dense core vesicle terminals nor type I synaptic glomeruli, assumed to be the endings of unmyelinated nociceptive terminals, were found on these interneurons. Moreover, there is no vesicular glutamate transporter 3-immunoreactive boutons, specific of C-LTMRs, on PKC $\gamma$ -immunoreactive interneurons. PKC $\gamma$ -immunoreactive interneurons contain GABA<sub>A</sub>ergic and glycinergic receptors. At subcellular level, PKC $\gamma$ -immunoreactivity is mostly concentrated on plasma membranes, close to, but not within, postsynaptic densities. That only myelinated primary afferents were found to contact PKC $\gamma$ -immunoreactive interneurons suggests that myelinated, but not unmyelinated, LTMRs play a critical role in the expression of mechanical allodynia.

## **KEY WORDS**

Medullary dorsal horn (MDH), protein kinase C gamma (PKC $\gamma$ ), C-low threshold mechanoreceptor (C-LTMR), vesicular glutamate transporter 3 (VGLUT3),  $\gamma$ -aminobutyric acid type A receptor (GABA<sub>A</sub>), glycine receptor, electron microscopy, confocal microscopy

## INTRODUCTION

Pain is initiated by peripheral tissue damage and inflammation (inflammatory pain) or lesions to the nervous system (neuropathic pain). It is characterized by persistent pain hypersensitivity, including spontaneous pain (pain experienced in the absence of any obvious peripheral stimulus), hyperalgesia (exacerbated response to noxious stimuli) and allodynia (pain in response to normally innocuous stimuli).

It is now well established that the development and maintenance of persistent pain hypersensitivity involves, at least in part, changes in the processing of sensory information within the dorsal horn (DH). Primary afferents terminate in DH with a distribution pattern that is determined by sensory modality: fine primary afferents, either lightly myelinated A $\delta$ - or unmyelinated C-fibers, including most nociceptors, innervate the more superficial laminae, I and outer II (II<sub>o</sub>), whereas touch-sensing, lightly myelinated D-hair A $\delta$ -fibers and heavily myelinated A $\beta$ -fibers terminate in lamina inner II (II<sub>i</sub>)-III and II<sub>i</sub>-V, respectively (Li et al., 2011; for review see: Basbaum et al., 2009). Under physiological conditions, informations from different modalities are kept separate. However, under conditions of disinhibition, A $\beta$ -fiber-mediated information can reach lamina I, nociceptive output neurons by way of a polysynaptic excitatory pathway (Torsney and MacDermott, 2006; Miraucourt et al., 2007). Activation of such local circuits has been shown to be associated with mechanical allodynia, leading to a miscoding of tactile information by cells that normally only detect painful stimuli (Miraucourt et al., 2007).

The  $\gamma$  isoform of protein kinase C (PKC $\gamma$ ) appears to play an important role in the development of mechanical allodynia. Increases in PKC $\gamma$  activity in spinal DH are triggered by both nerve (Miletic et al., 2000; Nakajima et al., 2011) and tissue injury (Martin et al., 1999; Cheng et al., 2008). Genetic impairment of PKC $\gamma$ , by the way of targeted deletion

(Malmberg et al., 1997) or RNA interference (Zou et al., 2011) decreases specifically the behavioral allodynia that occurs days after nerve injury but has no effect on normal acute pain responses. Similarly, pharmacological impairment of PKC $\gamma$  inhibits mechanical allodynia that is produced by glycine receptor antagonists (Miraucourt et al., 2007). That, in DH, PKC $\gamma$  is restricted to a population of local circuit interneurons in the lamina II<sub>i</sub> (Malmberg et al., 1997; Polgar et al., 1999) has led to the suggestion that these lamina II<sub>i</sub> interneurons participate in local circuits underlying mechanical allodynia. However, how activation of PKC $\gamma$  neurons engages the ‘pain’ transmission circuit, is still unclear.

In the present study, we used immunocytochemistry at the light and electron microscopy level to get insights into the neuronal function of lamina II<sub>i</sub> PKC $\gamma$ -immunoreactive interneurons in rats. We first characterized PKC $\gamma$  expression in the spinal trigeminal nucleus, mainly its most caudal subnucleus or medullary dorsal horn (MDH), which has received little attention (Li et al., 2001a; Li et al., 2001b), though it is a major relay nucleus for the somatosensory information from the face and meninges. We then addressed the general issue of synaptic inputs to lamina II<sub>i</sub> PKC $\gamma$  interneurons in the MDH. C-low-threshold mechanoreceptors (C-LTMR) are a subpopulation of unmyelinated, cutaneous sensory afferents that likely process pleasant and socially relevant aspects of touch (Loken et al., 2009; Olausson et al., 2010) and mechanical allodynia after inflammation or nerve injury (Seal et al., 2009). That, on the one hand, PKC $\gamma$  interneurons are specifically activated by tactile, but not noxious, inputs (Miraucourt et al., 2007; Neumann et al., 2008), and, on the other hand, in mice, C-LTMRs terminate in a very restricted DH area that precisely overlaps with the band of PKC $\gamma$ -immunoreactive interneurons in lamina II<sub>i</sub> (Seal et al., 2009; Li et al., 2011), raises the question as to whether C-LTMRs directly terminate on PKC $\gamma$ -immunoreactive interneurons. Fast inhibitory synaptic transmission within the DH is mediated

by both  $\gamma$ -aminobutyric acid (GABA)-ergic and glycinergic transmission (for review: see Zeilhofer et al., 2012). That dorsally directed local circuits underlying mechanical allodynia appear to be unmasked by selectively glycinergic receptor antagonists (Miraucourt et al., 2007; Miraucourt et al., 2009) raises the question as to whether GABA<sub>A</sub> receptors are also present on lamina II<sub>i</sub> PKC $\gamma$  interneurons. Therefore, we examined whether both glycinergic and GABA<sub>A</sub>ergic receptors are present on lamina II<sub>i</sub> PKC $\gamma$  interneurons. Finally, we examined the subcellular localization of PKC $\gamma$  within lamina II<sub>i</sub> interneurons in baseline conditions.

## **MATERIAL AND METHODS**

### **Animals**

Adult male Sprague–Dawley rats (250 - 275 g) were obtained from Charles River (L'Arbresle, France) and maintained in a controlled environment (light on 19.00 - 7.00, 22°C). Water and food were provided *ad libitum*. Rats were housed 3-4 per cage. All efforts were made to minimize the number of animals used. The experiments followed the ethical guidelines of the International Association for the Study of Pain and ethical guidelines of the Directive 2010/63/UE of the European Parliament and of the Council on the protection of animals used for scientific purpose.

### **Antibody characterization**

Details of the primary antibodies used in the present study are provided in Table 1.

### ***GABA<sub>A</sub> receptor***

GABA<sub>A</sub> receptor purified monoclonal antibody BD17 (IgG1) was raised in mouse and recognizes an epitope on the extracellular domain of the  $\beta$ 2 and  $\beta$ 3 subunits of the GABA<sub>A</sub> receptor. The immunogen was a GABA/benzodiazepine receptor purified from bovine cortex.



This antibody reveals two proteins bands corresponding to the  $\beta 3$  (57kDa) and  $\beta 2$  (55 kDa) subunits in Western blot analysis of both rat brain and spinal cord lysates (Fig. 1). Staining is eliminated by replacing primary antibodies with nonimmune serum (Fig. 1).

### ***Glycine receptor***

Glycine receptor  $\alpha$  purified monoclonal antibody mAb4a (IgG1) was raised in mouse and recognizes an epitope located within the amino acid residues 99-105 of the N-terminal region of the mature glycine receptor  $\alpha 1$ -subunit. The immunogen was a purified rat spinal cord glycine receptor. This antibody reveals the appropriate protein band (48 kDa) on Western blot analysis of rat spinal cord (Fig. 1). An additional fixation with a mixture of methanol:acetic acid (95:5) for 10 min at  $-20^{\circ}\text{C}$  was performed when using this antibody according to the manufacturer (Dumoulin et al., 2001).

### ***NeuN***

NeuN purified monoclonal antibody A60 (IgG1) was raised in mouse and apparently labels all spinal neurons in rats too (Todd et al., 1998). This antibody used in our experiments reveals a similar immunoreactivity pattern in the rat MDH (see Fig. 5) compared with DH (Todd et al., 1998). The immunogen was purified cell nuclei extracted from mouse brain. This antibody recognizes mostly nuclear antigens and weakly cytoplasm ones (Mullen et al., 1992; Wolf et al., 1996). This antibody reveals three bands in the 46-48 kDa region on Western blot analysis and binds to DNA *in vitro* (Mullen et al., 1992). It recognizes neither glia cells nor non neuronal tissue. No staining is observed when the primary antibody is replaced by normal serum in mouse (Lorenzo et al., 2008).

### ***PKC $\gamma$***

PKC $\gamma$  affinity purified polyclonal antibody (C-19) was raised in rabbit and recognizes an epitope located within the amino acid residues 679-697 at the C-terminal variable (V5) region

of the PKC $\gamma$ . A mouse synthetic peptide, Asp-Phe-Val-His-Pro-Asp-Ala-Arg-Ser-Pro-Thr-Ser-Pro-Val-Pro-Val-Pro-Val-Met, corresponding to these amino acid residues 679-697 of PKC $\gamma$  was used as immunogen. This antibody reveals two closely spaced bands of the appropriate molecular weight (80 kDa) on Western blot analysis of brain lysates (Cardell et al., 1998). Immunoreactivity is observed within the lamina II<sub>i</sub> of mouse DH (Malmberg et al., 1997), rat MDH (Fig. 2; Miraucourt et al., 2007) but not of PKC $\gamma$ <sup>-/-</sup> mouse DH (Malmberg et al., 1997). Staining is completely abolished when the antiserum is preabsorbed for 30 min with 40 ng.ml<sup>-1</sup> of the mouse PKC $\gamma$  peptide (Fig. 2A).

PKC $\gamma$  monoclonal antibody PK-G4 (IgG2a) was raised in mouse and recognizes an epitope located within the amino acid residues 684-697 at the C-terminal variable (V5) region of the PKC $\gamma$ . A synthetic peptide, Cys-Asp-Ala-Arg-Ser-Pro-Thr-Ser-Pro-Val-Pro-Val-Pro-Val-Met, corresponding to the amino acid residues 684-697 of PKC $\gamma$  was used as immunogen. This antibody reveals the band of the polypeptide PKC (80 kDa) on Western blot analysis of brain lysates in wild type but not in PKC $\gamma$ <sup>-/-</sup> mice (Yevseyenkov et al., 2009). It shows a unique immunostaining in lamina II<sub>i</sub> of MDH (Fig. 2C) where PKC $\gamma$  is known to be restricted (Miraucourt et al., 2007). Stainings with the monoclonal and polyclonal antibodies fully overlap (Fig. 2D).

### ***VGLUT3***

The vesicular glutamate transporter 3 (VGLUT3) polyclonal antibody was raised in guinea-pig and was generously provided by Dr. H. Hioki (Kyoto university, Japan). It was affinity-purified with antigen-conjugated column. This antibody recognizes an epitope located within amino acid residues 564-588 at the C-terminal region of the VGLUT3 antigen in mouse and rat brain tissue using immunoblotting and immunohistochemistry. It shows a unique immunostaining in the lamina II<sub>i</sub> of MDH (Fig. 7) where VGLUT3 is known to be restricted

(Seal et al., 2009). Using this antibody, no immunoreactivity is observed in VGLUT3<sup>-/-</sup> mice (Nakamura et al., 2004; Seal et al., 2009).

The specificity of antibodies was also verified by omitting either primary or secondary antibodies (data not shown). In those cases, no staining was visualized confirming the appropriate dilution and specificity of antibodies used in this study.

### **Protein extraction and Western blot**

Lumbar spinal cord or brain tissue were homogenized by sonication in ice-cold buffer (buffer RIPA containing 1% NP-40, 0.5% sodium Deoxycholate and 0.1% of Sodium Dodecylsulfate) containing protease inhibitors (Roche SAS, Boulogne-Billancourt, France). After 30 min at 4°C in rotating wheel, the mix was centrifuged at 14 000 rpm for 15 min. The supernatants were collected and quantified by the Lowry method (Bio-Rad DC Protein Assay, Marnes-la-Coquette, France).

Total proteins were subjected to SDS-PAGE electrophoresis on 10% polyacrylamide gels. Membranes were incubated overnight at 4°C with mouse anti-GABA<sub>A</sub> receptor antibody (1/500) or with mouse anti-Glycine receptor antibody (1/1000). Immunoreactivity was detected using polyclonal goat anti-mouse immunoglobulins-HRP (DakoCytomation, Glostrup, Denmark). Controls were incubated without primary antibody. Bands were visualized by using a chemiluminescent detection system (luminata, Millipore, Molsheim, France). Pictures of the blots were taken with a ChemiGenius 2XE apparatus under the control of GeneSnap program (Syngene, Cambridge, UK).

### **Immunohistochemistry**

Rats were deeply anaesthetized with urethane (1.5g/kg i.p.) and transcardially perfused with ice-cold 0.1 M phosphate buffer saline, pH 7.4 (PBS) followed by ice-cold 4%

paraformaldehyde (PFA) diluted in PBS. Brainstem was quickly removed and immersed into fixative solution for 2 h at 4°C before cryoprotection in 30% sucrose in PBS for 48 h at 4°C. Forty  $\mu\text{m}$  coronal sections were cut on a freezing microtome and collected in PBS before being processed. Free floating sections were first treated with 50 mM  $\text{NH}_4\text{Cl}$  diluted in PBS for 30 min at room temperature (RT). After several washes in 0.1 M PBS with 0.2% Triton (PBS-Tx), sections were blocked by pre-incubation in 5% Normal Goat Serum (NGS) in PBS-Tx for 1 h at RT. After washes in PBS-Tx, sections were incubated with a primary antibody diluted in 5% NGS in PBS-Tx for 24 h at 4°C. Sections were then washed with 5% NGS followed by washes in PBS-Tx. Tissues were incubated with secondary fluorescent antibody diluted in 5% NGS in PBS-Tx for 1 h at RT. Finally, sections were washed in 5% NGS, in PBS-Tx and then in PBS. Sections were transferred onto gelatinized slides before being coverslipped with DPX mountant for histology. Neurons containing VGLUT3, NeuN,  $\text{GABA}_A$  receptor, glycine receptor and/or  $\text{PKC}\gamma$  immunoreactivity in the MDH were first photographed using a fluorescent Zeiss Axioplan 2 Imaging microscope coupled with a Hamamatsu C4742–95 digital camera, by switching between FITC and Rhodamine filter sets at respectively x5 and x20 magnifications before being analyzed with a confocal microscope. The delineation of the MDH was based upon the Paxinos and Watson atlas (Paxinos and Watson, 1997) and our own myeloarchitectural atlas as determined by our previous work (Miraucourt et al., 2011).

For control of  $\text{PKC}\gamma$  antibody specificity, MDH sections were cut in contralateral and ipsilateral hemisections and incubated into either primary antibody preabsorbed with 40  $\text{ng}\cdot\text{ml}^{-1}$  of  $\text{PKC}\gamma$  synthetic peptide (Santa Cruz Biotechnology p-sc-211, CA) 30 min at RT or with primary antibody alone as described above.

### **Confocal laser scanning microscopy**

Sections were analyzed with a Zeiss LSM 510 confocal laser scanning microscope by using 488- and 543-nm excitation laser light. In order to suppress emission cross-talk, the microscope was configured to perform all scanning in sequential mode. Z-series were scanned at x20 and x63 magnifications with an oil immersion lens and a z-step of 0.37  $\mu\text{m}$ . The z-series were first examined with the Zeiss LSM image examiner<sup>®</sup>. Each image was then analyzed with Fiji-ImageJ 1.47 program (<http://rsbweb.nih.gov/ij> ; Schindelin et al., 2012). For fluorescence pattern analysis, a single image corresponding to the z-middle of cell was quantified using plot profile analysis plug-in. For this, a single line going from either side of the neuron through the plasma membrane, cytoplasm and nucleus was traced and grey values calculated. The relative changes in fluorescence intensity were measured from the background level and plasma membrane and cytoplasm separated considering the limited resolution of light. To ensure that the analyzed neurons were entirely included in the z-section, 3D-reconstruction was systematically performed using the 3D-viewer plug-in (Schmid et al., 2010) after cropping an isolated neuron.

### **Electron microscopy (pre-embedding method)**

Animals were deeply anesthetized with pentobarbital and perfused through the ascending aorta with an ice-cold fixative containing 4% PFA, 1.5% picric acid and 0.1% glutaraldehyde diluted in 0.1 M phosphate buffer, pH 7.4 (PB). Brainstem was rapidly dissected out and postfixed in the same fixative without glutaraldehyde for 2 h and then rinsed in 0.1 M PBS, pH 7.4 at 4°C overnight. Free-floating sections (40- $\mu\text{m}$  thickness) were obtained on a Vibratome (Leica, Nanterre, France) and permeabilized 30 min with 1% borohydride diluted in 0.1 M PBS. After several washes in 0.1 M PBS, sections were then saturated 30 min at RT with 1% Bovine Serum Albumin (BSA) diluted in 0.1 M Tris, 0.15 M NaCl and 0.05 % Tween (TNT). For single immunostaining, sections were first incubated overnight at 4°C with

the primary antibody diluted in TNT with 1% BSA. After several washes with TNT, sections were incubated for 2 h at RT with the secondary antibody diluted in TNT with 1% BSA followed by a Diaminobenzidine (DAB) reaction. For immunogold reaction, sections were pre-treated with 1% glutaraldehyde diluted in distilled water 10 min at RT followed by several washes with TNT. A silver amplification was performed with HQ SILVER Nanoprobes (Yaphank, NY, USA). In all cases, sections were post-fixed for 1 h in dark with 1% OsO<sub>4</sub> diluted in PB after several washes in PB. Sections were then dehydrated through a graded series of ethanol (10 min in 50% ethanol, 10 min in 70% ethanol, 2 x 15 min in 95% ethanol, 2 x 20 min in 100% ethanol and 2 x 30 min in propylene oxide). Sections were embedded by the progressive temperature lowering technique. Briefly sections were incubated for 2 h at RT with 50% EPON resin and 50% propylene oxide, overnight with 100% EPON resin, 4 h at RT with freshly-made EPON resin and then 36 h at 60 °C. Ultrathin sections (60–100 nm) from blocs were mounted on gold mesh grids and observed under a HITACHI H7650 electron microscope. For double immunostainings, DAB reaction was performed immediately after Silver amplification and several washes with distilled water.

For sampling, lamina II<sub>i</sub> containing embedded blocs were first cut by visualizing the dark PKC $\gamma$  immunoreactive band formed by DAB reaction. Selected blocs were then mounted onto an ultramicrotome to be cut in ultrathin sections. For sections stained by gold particles, no visual immunoreactivity could be used to select lamina II<sub>i</sub>. Therefore, embedded blocs were first cut according to the relative distance of lamina II<sub>i</sub> to the Lissauer tract ( $\approx$  65  $\mu$ m) before being mounted onto an ultramicrotome to be cut in ultrathin sections. Serial sections were mounted onto gold mesh grids, each grid containing a distant focal plane. Only the most immunoreactive ultrathin section was selected in each grid to avoid repetitive analysis. Because the number of immunoreactive profiles can vary from one ultrathin section to

another, the number of sections used for each rat was determined so that the number of immunoreactive profiles was similar in the three independent rat samples.

### **Cell counting**

NeuN and/or PKC $\gamma$  immunoreactive cells in the MDH were first scanned at x20 magnification and analyzed using Fiji-ImageJ 1.47 program. Sections were analyzed at -1200  $\mu\text{m}$  from the obex aperture, i.e. at about the middle of the rostro-caudal extend of MDH (Strassman and Vos, 1993; Miraucourt et al., 2011), for each rat (n=4). For cell counting, a 300 x 400  $\mu\text{m}$  window was positioned so that its median axe was perpendicular to the MDH border and its dorsolateral end at the border between the white and grey matter (Lorenzo et al., 2008). In addition, the center of this window was on the diagonal of a rectangle enclosing the whole MDH (see Fig. 3). NeuN and PKC $\gamma$  immunoreactivity profiles were counted separately using the cell counter plug-in. Delimitation of laminae borders was first done using merged NeuN/PKC $\gamma$  images. The border between lamina I and lamina II<sub>o</sub> was delimited so that the lamina I contained the apparent unicellular layer of characteristic large lamina I output neurons. The border between lamina II<sub>o</sub> and lamina II<sub>i</sub> was set based on the apparition of a dense PKC $\gamma$  immunoreactive neuropil and that between lamina II<sub>i</sub> and III<sub>o</sub> on the disappearance of this neuropil. As we did not use neurochemical markers to delineate the border between lamina III and IV (Polgar et al., 2006), we did not separate these two last laminae. A cell was counted as immunopositive if (1) immunostaining of a single cell was clearly defined, (2) its level was at least two times that of the background levels, and (3) it had disappeared at both the top and bottom surfaces of z-stack. To ensure that a cell was not counted several times, the immediate look-up z-section was used as reference and compared to the subsequent z-section. 3D reconstruction using the 3D-viewer plug-in was also used to compare our counting with the representative neuronal structure reconstructed from the z-

series. If any doubt was present, cells were reanalyzed at a higher magnification using x63 oil immersion lens.

### **Data and statistical analysis**

Rostro-caudal levels of the MDH are shown as distance in  $\mu\text{m}$  from the obex aperture. VGLUT3-immunoreactive fibers were analyzed using Fiji-ImageJ 1.47 program and the simple neurite tracer plug-in (Longair et al., 2011) in superficial layers of the MDH within the section -1200  $\mu\text{m}$  from the obex aperture. Because (1) the intensity of immunoreactivity can vary from one sample to another and (2) lamina width is not even in the MDH, we did not assess fluorescence intensity in each lamina. Rather, pictures from neurolucida reconstructions were digitized. The area of VGLUT3 immunoreactivity was quantified using Fiji-ImageJ 1.47 program and normalized to the total area of the respective lamina. For electron microscopy, immunogold particles were analysed using Fiji-ImageJ 1.47 program and PointDensity or DistToPath plug-in as previously described (Larsson et al., 2001). Distances between the midpoint of each gold particle signaling GABA<sub>A</sub> receptor, glycine receptor or PKC $\gamma$  immunoreactivity and the centre line of the associated plasma membrane or the middle of the synaptic cleft were measured. Areal densities (number of particles per  $\mu\text{m}^2$ ) were also computed. Subcellular compartmentation of PKC $\gamma$ -immunoreactivity in MDH and cerebellar neurons was normalized to the total fluorescence of the cell. Values are expressed as mean  $\pm$  SEM. All quantitative analyses, graphs and statistical tests were performed on GraphPad Prism<sup>TM</sup>, version 5.0. For statistical analyses, we used a one-way ANOVA followed by a Tuckey *post hoc*, after passed the Kolmogorov-Smirnov normality test. In all cases,  $p < 0.05$  was considered as statistically significant. Pictures were optimized for visual quality using Fiji-ImageJ 1.47 program at the end of analysis. Figures were made using CorelDRAW<sup>©</sup> 12.



## RESULTS

### Distribution of PKC $\gamma$ interneurons within the spinal trigeminal nucleus

In the spinal trigeminal nucleus, PKC $\gamma$  immunoreactivity was found exclusively within the MDH (Fig. 4B-E) where it formed a continuous, homogeneous and dense band in superficial laminae from the dorsomedial to the ventrolateral part in transverse sections. Actually, such PKC $\gamma$  immunoreactive band varied throughout the rostro-caudal axis of the MDH. Rather large at the caudal end of the MDH (thickness at -2400  $\mu\text{m}$ :  $65.6 \pm 2.3 \mu\text{m}$ ,  $n = 5$ ), this band progressively narrowed (-800  $\mu\text{m}$ :  $36.1 \pm 0.7 \mu\text{m}$ ,  $n = 5$ ), became scattered (between -400 and 0  $\mu\text{m}$ ; corresponding to the transition from the MDH to subnucleus interpolaris (Sp5I), and finally vanished (at +400  $\mu\text{m}$ ), where both MDH and Sp5I overlap and laminar organization has disappeared (Fig. 4A).

At low magnification, PKC $\gamma$  immunoreactivity appeared as mainly a dense neuropil with few strongly immunoreactive cell bodies and associated dendrites in lamina II<sub>i</sub>. However, though most immunoreactive neurons were located within this lamina II<sub>i</sub> neuropil, there were also stained cells in lamina I, lamina II<sub>o</sub>, and outer lamina III (Fig. 5B, D). As previously observed (Polgar et al., 1999), PKC $\gamma$  immunoreactivity was weaker in the more dorsal (lamina I and II<sub>o</sub>) than in the more ventral cells (lamina II<sub>i</sub> and III). When sections immunostained with both PKC $\gamma$  and NeuN were viewed at high magnification (Fig. 5C), the vast majority of PKC $\gamma$  immunoreactive cells ( $92 \pm 3\%$ ) were found to be NeuN-positive, indicating that these were neurons.

Quantitative analysis of NeuN immunoreactivity indicates that lamina II exhibits the most intense neuronal density, especially at its dorsal part ( $47 \pm 4$  and  $34 \pm 4$  neurons /100  $\mu\text{m}^2$  in lamina II<sub>o</sub> and II<sub>i</sub>, respectively, compared with  $14 \pm 4$  and  $24 \pm 2$  neurons /100  $\mu\text{m}^2$ , in lamina I and III-IV, respectively) as already observed in spinal DH (Todd et al., 1998; Polgar et al.,

2004) (Fig. 5A). Most of PKC $\gamma$ -immunoreactive neurons were located within lamina II<sub>i</sub> ( $52 \pm 6\%$ ) where they represent  $30 \pm 5\%$  of the total number of neurons within this lamina (Fig. 5D, E). Results of this study are shown in Table 4. As PKC $\gamma$  immunoreactivity is predominantly found in lamina II<sub>i</sub>, we will focus on PKC $\gamma$ - immunoreactive interneurons in lamina II<sub>i</sub> of the MDH in the remainder of the paper.

### **Synaptic inputs to lamina II<sub>i</sub> PKC $\gamma$ interneurons**

We performed a transmission electron microscopy study of synapses on PKC $\gamma$ -immunoreactive interneurons. A total 199 synaptic appositions were analyzed, 46 on somatic and 153 on dendritic PKC $\gamma$ -immunoreactive profiles. Most of them (67%) were asymmetric (likely excitatory), almost exclusively (96%) axo-dendritic synapses, whereas 33% were symmetric (likely inhibitory), mostly axo-somatic ones (62%) (Fig. 6 and Table 5). Out of the 128 analyzed axo-dendritic asymmetric synapses on PKC $\gamma$  immunoreactive interneurons, only 20 (16%) were between large primary afferent terminals and these neurons (Fig. 6 and Table 5).

In mice, C-LTMRs have been shown to specifically express the vesicular glutamate transporter 3 (VGLUT3) and terminate in lamina II<sub>i</sub>, precisely within the PKC $\gamma$  immunoreactive band (Seal et al., 2009; Li et al., 2011). To examine whether C-LTMRs directly terminate on PKC $\gamma$  immunoreactive neurons, we performed a light and electron microscopy double labeling study of MDH sections with VGLUT3 and PKC $\gamma$  antibodies. Our light microscopy study confirmed that, in rats too, VGLUT3 immunoreactive fibers terminate mostly in lamina II<sub>i</sub>. They travel from the Lissauer tract to lamina II<sub>i</sub> in a dorso-ventral direction and form a dense plexus within this lamina where PKC $\gamma$  immunoreactivity is predominantly located (Fig. 7A-C). Indeed, VGLUT3 immunoreactivity was significantly higher in lamina II<sub>i</sub> compared to lamina I ( $p < 0.001$ ,  $n = 5$ ), II<sub>o</sub> ( $p < 0.001$ ,  $n = 5$ ) and III<sub>o</sub> ( $p <$

0.01, n = 5) (Fig. 7C). This raises the question as to whether VGLUT3-immunoreactive fibers form synapses onto PKC $\gamma$  interneurons. To address this question, altogether, 17 somatic and 167 dendritic PKC $\gamma$  immunoreactive profiles in 3 rats were analyzed at the electron microscopy level. Somewhat surprisingly, though, no synaptic contact between VGLUT3 immunoreactive terminals and PKC $\gamma$ -immunoreactive interneurons could be found. Finally, we observed only one morphological type of primary afferents terminals onto PKC $\gamma$ -immunoreactive neurons. Out of the 20 afferents that terminated onto PKC $\gamma$ -immunoreactive interneurons, we quantitatively analyzed 15 terminals that could be entirely observed and clearly delineated under the electron microscopy. These were large terminals (section area:  $2.55 \pm 0.43 \mu\text{m}^2$ , n = 15) with light axoplasm containing loosely packed, uniformly clear vesicles and numerous mitochondria ( $3.64 \pm 0.29$  per  $\mu\text{m}^2$ , n = 15) and forming the central element of synaptic glomeruli of type II (Ribeiro-da-Silva and Coimbra, 1982) (Fig. 7D). Such terminals are thought to be endings of myelinated primary afferent fibers (Ribeiro-da-Silva and Coimbra, 1982; Maxwell and Réthelyi, 1987; Larsson and Broman, 2005; Neumann et al., 2008). Interestingly, neither large dense core vesicle terminals nor synaptic glomeruli of type I, which are assumed to be the endings of unmyelinated peptidergic and IB4 primary afferent terminals, were found on PKC $\gamma$ -immunoreactive interneurons. Together with our failure to find any VGLUT3-immunoreactive boutons on PKC $\gamma$ -immunoreactive interneurons, these observations strongly suggest that neither nociceptive nor tactile unmyelinated C-fibers terminals terminate on lamina II<sub>i</sub> PKC $\gamma$  immunoreactive neurons.

It was recently shown that PKC-immunoreactive interneurons receive direct glycinergic synaptic inhibition (Miraucourt et al., 2007). Do lamina II<sub>i</sub> PKC $\gamma$  interneurons receive pure glycinergic synaptic inhibition? To address this question, we assessed glycinergic and GABA<sub>A</sub>ergic receptor immunoreactivity on lamina II<sub>i</sub> PKC $\gamma$ -immunoreactive interneurons, at the electron microscopic level. Altogether, 7 somatic and 20 dendritic PKC $\gamma$ -immunoreactive

profiles were analyzed for GABA<sub>A</sub> receptor immunoreactivity and an additional 7 somatic and 20 dendritic PKC $\gamma$ -immunoreactive profiles, for glycine receptor immunoreactivity. The sizes of these PKC $\gamma$ -immunoreactive somatic and dendritic profiles were the same in the two series of experiments:  $33.7 \pm 4.0 \mu\text{m}^2$  and  $2.5 \pm 0.6 \mu\text{m}^2$  compared with  $42.5 \pm 5.9 \mu\text{m}^2$  and  $3.2 \pm 1.1 \mu\text{m}^2$  for GABA<sub>A</sub> and glycine receptor-immunoreactive somatic and dendritic profiles, respectively. Altogether, 433 gold particles signaling GABA<sub>A</sub> receptor immunoreactivity (Fig. 8) and 515 ones signaling glycine receptor immunoreactivity (Fig. 9) were registered on PKC $\gamma$ -immunoreactive interneurons. GABA<sub>A</sub> or glycine receptor immunoreactivity was found on all PKC $\gamma$ -immunoreactive somas (Fig. 8A and 9A) and most, but not all, PKC $\gamma$ -immunoreactive dendrites. Indeed, no gold particle for GABA<sub>A</sub> and glycine receptor immunoreactivity could be registered on 7 (Fig. 8E) and 6 (Fig. 9E) PKC $\gamma$ -immunoreactive dendrites, respectively. A quantitative analysis was performed to investigate the precise distribution of gold particles signaling GABA<sub>A</sub> and glycine receptor immunoreactivities to the plasma membranes of PKC $\gamma$ -immunoreactive interneurons (Fig. 8B-E and 9B-E). This distribution indicates that GABA<sub>A</sub> and glycine receptors are localized primarily to plasma membranes and the underlying cytoplasm. About half of gold particles signaling GABA<sub>A</sub> and glycine receptor immunoreactivities are found within 400 nm of somatic (Fig. 8B,C and 9B,C) and 100 to 150 nm of dendritic (Fig. 8D and 9D) plasma membranes. Consistent with this, when gold particles signaling GABA<sub>A</sub> and glycine receptor immunoreactivities are registered on PKC $\gamma$ -immunoreactive dendrites, the areal density of gold particles in dendrites is higher than that in somas (Fig. 8E and 9E). It is noteworthy that GABA<sub>A</sub> and glycine receptor immunoreactivities were also found within cytoplasm and, to a lesser extent, nuclei. This particular localization of inhibitory receptors suggests a dynamic expression at the neuronal membrane with their different states (active, biosynthesized or recycled).

## **Subcellular localization of PKC $\gamma$ immunoreactivity within lamina II<sub>i</sub> interneurons**

PKC $\gamma$  primary antibody combined with HRP-conjugated secondary antibody revealed two types of morphologically distinct PKC $\gamma$ -immunoreactive interneurons: fusiform and oval-shaped (Fig. 10A, B). The size of their soma, measured by confocal microscopy, is  $13.1 \pm 0.8 \times 6.5 \pm 0.3$  and  $9.1 \pm 0.4 \times 7.4 \pm 0.4$   $\mu\text{m}$  for fusiform ( $n = 10$ ) and oval-shaped ( $n = 10$ ) neurons, respectively. Immunoreactivity is found in the somatic and dendritic cytoplasm and, to a lesser extent, in the nucleus. At high magnification, though DAB reaction product can be found throughout the cytoplasm, it appears to be localized primarily to, or close to, the plasma membrane (Fig. 10A, B). This suggests that PKC $\gamma$  is mostly associated with plasma membranes.

To further assess the subcellular distribution of PKC $\gamma$ , we used the quantitative electron microscopy immunogoldcytochemistry. Altogether, 267 particles signaling PKC $\gamma$  were registered on 8 dendrites (3 rats) (Fig. 10 C, D), being exclusively postsynaptic (see examples in Fig. 10 C, E). A first quantitative analysis was performed to assess the distribution of gold particles signaling PKC $\gamma$  immunoreactivity to plasma membranes (Fig. 10 D). The density of gold particles peaks at 30 nm from plasma membranes. Thus,  $56.6 \pm 6.6\%$  of gold particles are within 30 nm of plasma membranes (corresponding to the experimentally determined maximum epitope–gold particle–centre distance (Matsubara et al., 1996)) and  $77.2 \pm 5.3\%$  within 50 nm. A second quantitative analysis examined the distribution of gold particles signaling PKC $\gamma$  immunoreactivity to synaptic active zones (Fig. 10 F). Gold particles appear to preferentially cluster close to, but not within, the synaptic space (distance from synaptic clefts:  $248 \pm 11$  nm; 182 particles, 15 synapses, 3 rats) (Fig. 10 E, F). Moreover, presumably endosomal vesicles highly stained with gold particles, were often found close to the synaptic space (see examples in Fig. 10 C, E).

Most gold particles for PKC $\gamma$  immunoreactivity are closely associated with plasma membranes in lamina II<sub>i</sub> interneurons. It is now well established that activation of glutamate receptors and the consecutive increase in cytosolic free Ca<sup>2+</sup> promotes a quick translocation of PKC for the cytoplasm to plasma membranes (Vaccarino et al., 1987; Sakai et al., 1997; Oancea et al., 1998; Oyasu et al, 2008). This raises the question as to whether the preferential localization of PKC $\gamma$  to plasma membranes actually results from a redistribution of the enzyme following glutamate release during the initiation of the perfusion procedure. To address this question, we compared the subcellular localization of PKC $\gamma$  immunoreactivity in MDH PKC $\gamma$ -immunoreactive interneurons with that in cerebellar Purkinje cells, which are known to also highly express PKC $\gamma$ . In baseline conditions, PKC $\gamma$  is located within the cytoplasm of these Purkinje cells and translocates to plasma membranes upon activation (Sakai et al., 1997; Sakai et al., 2004). Using light microscopy, we assessed the sub-cellular localization of PKC $\gamma$ -immunoreactivity in both trigeminal PKC $\gamma$ -immunoreactive interneurons (n = 10) and cerebellar Purkinje cells (n = 10) in naive animals. In MDH neurons, PKC $\gamma$  immunoreactivity is mainly located in plasma membranes (Fig. 11A, empty arrow head) and to a lesser extent in the cytoplasm (Fig. 11A, filled arrowhead) and nucleus (Fig. 11A, arrow). On the other hand, PKC $\gamma$  immunoreactivity in cerebellar Purkinje cells is equally located in the cytoplasm (Fig. 11C, filled arrowhead) and plasma membranes (Fig. 11C, empty arrow head) and little, if none, in the nucleus (Fig. 11C, arrow). Quantitative analysis of gray levels in the different compartments of both trigeminal and cerebellar PKC $\gamma$  immunoreactive cells, using confocal microscopy, confirmed that PKC $\gamma$  immunoreactivity is predominantly located in plasma membranes in trigeminal interneurons (Fig. 11 B, E, F), but in both plasma membranes and cytoplasm, in cerebellar Purkinje cells (Fig. 11 D, E, G). These results thus suggest that the localization of PKC $\gamma$  on plasma membranes in baseline conditions is a specific feature of trigeminal interneurons. However, clearly, trigeminal

PKC $\gamma$ -immunoreactive neurons and cerebellar Purkinje cells exhibit many other morphological differences including size and nucleus/cytoplasm ratio (Fig. 11 B, D).

## DISCUSSION

Using light and electron microscopy, our anatomical and morphological study shows that PKC $\gamma$  immunoreactivity is restricted to a single subnucleus in the spinal trigeminal nucleus: the MDH. Within MDH, PKC $\gamma$ -immunoreactive neurons are concentrated in superficial laminae, mostly in lamina II<sub>i</sub>, where they constitute 1/3 of the total number of neurons. The majority of synapses on lamina II<sub>i</sub> PKC $\gamma$ -immunoreactive interneurons are asymmetric (likely excitatory). However, neither large dense core vesicle-containing boutons nor VGLUT3-immunoreactive ones, which are both specific of unmyelinated afferents, could be found forming contacts with lamina II<sub>i</sub> PKC $\gamma$ -immunoreactive interneurons. Lamina II<sub>i</sub> PKC $\gamma$ -immunoreactive interneurons receive both GABA<sub>A</sub>ergic and glycinergic inputs. At the subcellular level, PKC $\gamma$  immunoreactivity within lamina II<sub>i</sub> MDH neurons is mostly concentrated on plasma membranes, close to (but not within) postsynaptic densities, in baseline conditions. Such subcellular localization is thus very different from that in Purkinje cells where PKC $\gamma$  immunoreactivity is mostly cytoplasmic in the same conditions.

### Technical considerations

We used a modification of the physical dissector method (Williams and Rakic, 1988; Coggeshall, 1992; Guillery, 2002; Polgar et al., 2004; Polgar et al., 2011) to provide an unbiased count of neurons. However, MDH is a heterogeneous structure which can vary in length and width throughout its rostro-caudal axis. A precise estimate of the total number of neurons that exist in this structure is therefore not possible. Nevertheless, to allow comparison with other studies, we report here densities of neurons in each MDH lamina at level -1200  $\mu\text{m}$ .

As previously described both in the spinal (Mori et al., 1990; Malmberg et al., 1997; Polgar et al., 1999) and medullary DHs (Li et al., 2001b; Miraucourt et al., 2007), PKC $\gamma$  immunoreactivity forms a dense plexus – consisting of cell bodies and associated dendrites within lamina II<sub>i</sub>. In addition, as in spinal DH (Polgar et al., 1999), PKC $\gamma$ -immunoreactive cells can also be found within lamina I, II<sub>o</sub> and III in the MDH. Nevertheless, PKC $\gamma$ -immunoreactive cells within both lamina II<sub>o</sub> and III are actually located close to the borders of lamina II<sub>i</sub>. Li et al. (2001b) reported that, in MDH, PKC $\gamma$ -immunoreactive cells are most prominent in lamina II, but in its outer part. The apparent discrepancy in the laminar localization of the band of strong PKC $\gamma$  immunoreactivity between this and our study probably reflects a slight difference in the placing of the lamina II/III border between the two studies.

### **PKC $\gamma$ neuronal population**

Double immunostaining with PKC $\gamma$  and NeuN antibodies reveals that the great majority of PKC $\gamma$  immunoreactive cells are also NeuN-positive indicating that they are neurons. That some PKC $\gamma$  immunoreactive cells are NeuN-negative raises the possibility that few PKC $\gamma$  immunoreactive cells are not neurons. This is very unlikely as PKC $\gamma$  has been shown to be expressed solely in neurons (Kose et al., 1988). Furthermore, no PKC $\gamma$  immunoreactive glia cells were found at the electron microscopy level in our study. Finally, it has already been noted that some neurons might be NeuN-negative (Lorenzo et al., 2008).

Cell counts reveal that about half of PKC $\gamma$ -immunoreactive neurons within MDH are restricted to lamina II<sub>i</sub> where they constitute 1/3 of the total number of neurons. Thus, taken together with previous estimates that the great majority of PKC $\gamma$ -immunoreactive neurons are excitatory (Martin et al., 1999; Polgar et al., 1999) and that nearly 70% of neurons in lamina II<sub>i</sub> are excitatory (Polgar et al., 2003), the present study indicates that PKC $\gamma$ -immunoreactive neurons make up around 50% of the excitatory neurons in lamina II<sub>i</sub>. Lamina II<sub>i</sub> PKC $\gamma$ -



immunoreactive interneurons would thus constitute one of the relatively largest population of neurons within one lamina of the medullary, and likely spinal, DHs. Preprotachykinin B (PPTB) immunoreactive neurons have also been shown to be highly concentrated in lamina II<sub>i</sub> where they constitute 16% of the neurons (Polgar et al., 1999). However most of PPTB immunoreactive neurons (76%) also express PKC $\gamma$  suggesting that these are the same neurons.

PKC $\gamma$  immunoreactivity varies between cells with most lamina II<sub>i</sub> PKC $\gamma$ -immunoreactive neurons being more immunoreactive than those within other laminae, in both the medullary (present results) and spinal DHs (Polgar et al., 1999). Several subpopulations of PKC $\gamma$ -immunoreactive neurons can be identified based on the intensity of PKC $\gamma$  immunoreactivity and expression of various neurochemical markers. For example, PKC $\gamma$ -immunoreactive neurons which express PPTB are weakly to moderately PKC $\gamma$  immunoreactive (Polgar et al., 2006) whereas those expressing neurotensin and somatostatin are moderately to strongly PKC $\gamma$  immunoreactive (Polgar et al., 1999). Since neurotensin and somatostatin are expressed in different sets of excitatory interneurons (Polgar et al., 1999; Polgar et al., 2006), neurotensin- and somatostatin-expressing PKC $\gamma$ -immunoreactive neurons may represent two different populations of PKC $\gamma$ -immunoreactive neurons. Similarly, AMPA receptor subunit glutamate 2/3 is expressed by only 50% and calbindin, by 15-25% of PKC $\gamma$ -immunoreactive neurons (Hughes et al., 2008). Moreover, a basic distinction can be drawn between PKC $\gamma$ -immunoreactive neurons within and outside lamina II<sub>i</sub>. Thus, AMPA receptor subunit glutamate 1 are present on 1/3 of PKC $\gamma$ -immunoreactive neurons in lamina I and II<sub>o</sub> but on very few in lamina II<sub>i</sub> (Hughes et al., 2008). Similarly, PKC $\gamma$ -immunoreactive neurons in lamina I and III can express NK1 receptors while there is essentially no co-expression of PKC $\gamma$  and NK1 receptor in lamina II<sub>i</sub> (Polgar et al., 1999; Hughes et al., 2008). This last

distinction might be associated with particular functional types as PKC $\gamma$  immunoreactive neurons in lamina I and III, but not in lamina II, can project to the thalamus (Li et al., 2001b). Therefore, the differential expression of PKC $\gamma$  immunoreactivity and the detection of various neurochemical markers strongly suggest that there are different subpopulations of PKC $\gamma$ -immunoreactive neurons with, likely, different functions. We therefore concentrated on PKC $\gamma$ -immunoreactive interneurons in lamina II<sub>i</sub> for the rest of our study.

### **Primary afferent inputs to PKC $\gamma$ -immunoreactive interneurons**

In mice, C-LTMR, terminate in lamina II<sub>i</sub>, precisely within the PKC $\gamma$  immunoreactive band (Seal et al., 2009; Li et al., 2011). The present study confirms that, in rats, too, VGLUT3 immunoreactivity is concentrated within lamina II<sub>i</sub> where about half of PKC $\gamma$  interneurons are located. Nevertheless, at the electron microscopic level, despite our efforts, no bouton containing VGLUT3 could be found forming synapse with PKC $\gamma$ -immunoreactive interneurons. It is then more likely that VGLUT3 containing afferents actually terminate on another population of lamina II<sub>i</sub> interneurons, namely calbindin-positive interneurons, which have been shown to receive unmyelinated, but not myelinated, primary afferents (Braz and Basbaum, 2009). It has to be noted, however, that C-LTMRs and PKC $\gamma$  interneurons appear to be both involved in injury- or inflammation-induced secondary mechanical allodynia, the former transmitting the input that produces pain (Malmberg et al., 1997; Seal et al., 2009), suggesting that they are somehow functionally related.

Together with our failure to find any large dense core vesicle-containing terminals on PKC $\gamma$  immunoreactive interneurons, these results suggest that PKC $\gamma$  interneurons do not receive any unmyelinated but rather exclusively myelinated primary afferents. Such conclusion is consistent with previous evidence showing that central terminals that conduct in the A $\beta$  range (Hughes et al., 2003) or contain the VGLUT1, the vesicular glutamate

transporter that is associated with large-diameter afferents (Neumann et al., 2008), contact PKC $\gamma$ -immunoreactive interneurons in lamina II<sub>i</sub>. In addition, a significant number of PKC $\gamma$ -immunoreactive interneurons in lamina II<sub>i</sub> are labeled by the transneuronal tracer wheat germ agglutinin when it is expressed in myelinated sensory neurons (Neumann et al., 2008; Braz and Basbaum, 2009). It has to be noted, however, that another type of LTMRs, namely thinly myelinated A $\delta$  D-hair, terminates in lamina II<sub>i</sub> and the outer part of lamina III (Maxwell and Réthelyi, 1987; Li et al., 2011). Since both A $\beta$  and A $\delta$  can form synaptic type II glomeruli in DH, our study cannot exclude the possibility that D-hairs directly contact PKC $\gamma$ -immunoreactive interneurons. Interestingly, activation of these two LTMRs leads to different pain symptoms (Treede and Magerl, 2000). In conclusion, different types of low threshold mechanoreceptors might thus engage different types of lamina II<sub>i</sub> neurons, the myelinated ones, the PKC $\gamma$  interneurons and the unmyelinated ones, the calbindin interneurons.

### **Inhibitory receptors onto PKC $\gamma$ -immunoreactive interneurons**

Our electron microscopic study shows that both glycine and GABA<sub>A</sub> receptors are expressed in lamina II<sub>i</sub> PKC $\gamma$  interneurons in adult rats. Evidence for glycine receptor immunoreactivity on lamina II<sub>i</sub> PKC $\gamma$ -immunoreactive interneurons had already been obtained (Miraucourt et al., 2007). The present study demonstrates in addition that GABA<sub>A</sub> receptors are also present on lamina II<sub>i</sub> PKC $\gamma$ -immunoreactive interneurons. This is consistent with anatomical evidence showing that lamina II<sub>i</sub> PKC $\gamma$  interneurons receive many vesicular GABA transporter-containing boutons, including those from neuropeptide Y GABAergic interneurons (Polgar et al., 2011). The electrophysiological observation that pure glycine receptor- and pure GABA<sub>A</sub> receptor-mediated events can be recorded in the same lamina II neuron and the fact that benzodiazepine can convert many pure glycine receptor-mediated events into mixed glycine receptor-GABA<sub>A</sub> receptor-mediated events (Keller et al., 2001)

suggests the existence of a heterogeneous junctional distribution of glycinergic and GABA<sub>A</sub>ergic receptors in lamina II neurons. However, because of technical limits including an overlap between electron-dense DAB product and gold particles in the plasma membrane due to a pre-embedding approach used in this study, we cannot address the issue of the pattern of distribution of glycinergic and GABA<sub>A</sub>ergic receptors in symmetric synapses formed onto lamina II<sub>i</sub> PKC $\gamma$ -immunoreactive interneurons. Also, it has to be noted that we might quantitatively underestimate the presence of both GABA<sub>A</sub> and glycine receptors at the cell membrane, including the synaptic cleft. Indeed, using a pre-embedding approach, monoclonal antibodies raised against the extracellular domain of GABA<sub>A</sub> and glycine receptors might not have reached the epitope because the narrow extracellular space preventing either the primary antibody or the 1.4-nm gold particle-coupled secondary antibody to enter the synaptic cleft (Zerari et al., 1998).

### **Subcellular localization of PKC $\gamma$**

PKC $\gamma$ , a conventional PKC isoform, is known to be translocated from the cytosol to plasma membranes when activated by Ca<sup>2+</sup> and diacylglycerol (Sakai et al., 1997; Oancea et al., 1998). Therefore, the translocation of PKC $\gamma$  can be considered as a good marker of whether this enzyme is activated or not. Our electron microscopic study of the subcellular localization of PKC $\gamma$  immunoreactivity in lamina II<sub>i</sub> interneurons of the MDH suggests that the enzyme is mostly located on, or very close to, plasma membranes. This result is consistent with previous electron microscopic studies in lamina II<sub>i</sub> neurons within the spinal DH pointing out that, PKC $\gamma$  immunoreactivity seems to be located on cell membranes in basal conditions, the reaction products being concentrated against the inner plasma membrane of dendrites (Mori et al., 1990; but see Martin et al., 2001). Since our result at the electron microscopic level showing that PKC $\gamma$  immunoreactivity, by analysis of DAB reaction product

or immunogold particles, is predominantly located on plasma membranes in lamina II<sub>i</sub> neurons implies that, in baseline conditions, most of the enzyme is activated, it was important to verify that this was not an experimental artifact. We performed two series of controls. We first investigated the subcellular localization of PKC $\gamma$  immunoreactivity in lamina II<sub>i</sub> interneurons using another technique, the confocal laser scanning fluorescent microscopy, and, then, compared it with that of Purkinje cells from the very same animals. On confocal images of PKC $\gamma$  immunoreactivity in lamina II<sub>i</sub> interneurons, fluorescence was predominantly located on plasma membranes. Interestingly, these images appear to be very similar to those of PKC $\gamma$  immunostaining previously obtained in spinal DH where the most intense staining is clearly found at the membrane level (see Fig. 5-6 in Polgar et al., 1999 or Fig. 8-9 in Polgar et al., 2011). Moreover, using the very same confocal microscopy, we assessed PKC $\gamma$  immunoreactivity in Purkinje cells from the same animals. Fluorescence appears to be rather uniformly distributed in the cytoplasm and plasma membranes, much the same as that of PKC $\gamma$  fused with green fluorescent protein of transgenic mice before any stimulation (Sakai et al., 2004). This suggests that the preferential localization of PKC $\gamma$  immunoreactivity on the plasma membranes of lamina II<sub>i</sub> interneurons is a special feature of these neurons, both in medullary and spinal DHs. However, Cardell et al. (1998), using high resolution immunogold cytochemistry, found that, in Purkinje cells too, a large proportion of gold particles for PKC $\gamma$  immunoreactivity is associated with plasma membranes. Nevertheless, comparison of the results obtained with fluorescent and electron-microscopy suggests that the preferential localization of PKC $\gamma$  immunoreactivity on the plasma membranes of lamina II<sub>i</sub> interneurons is a special feature of these neurons, both in medullary and spinal DHs. Nevertheless, it is noteworthy that the distance between the antibody-antigene site and gold particles can vary up to 30 nm (see Matsubara et al., 1996), and even more when using silver amplification.

Therefore, we cannot exclude the possibility that PKC $\gamma$  is not located within but rather very close to plasma membranes.

PKC $\gamma$  immunoreactivity, postsynaptic to asymmetric (excitatory) synapses, appears to cluster in the perisynaptic space at a distance of about 250 nm to the synaptic zone. Such distance corresponds to that of “synaptic molecules” (see Tardin et al., 2003), suggesting that PKC $\gamma$  contributes to synaptic functions. Thus, PKCs appear to be involved in horizontal and vertical membrane-trafficking of glutamate receptors in many brain areas including DH (Li et al., 1999; Groc et al., 2004). And PKC $\gamma$  itself has been shown to regulate synaptic plasticity in hippocampus (Abeliovich et al., 1993) as well as DH (Martin et al., 2001; Woolf, 2011).

In conclusion, our anatomical study establishes that PKC $\gamma$  immunoreactivity is restricted to a single subnucleus within the spinal trigeminal nucleus: the MDH. No PKC $\gamma$  immunoreactivity could be detected in the two other, more rostral, spinal trigeminal subnuclei: interpolaris and oralis. It is interesting to note that these three subnuclei exhibit very different anatomical organizations: only MDH includes laminae, much the same as those in spinal DH, and receives unmyelinated C-fiber primary afferents (Dallel et al., 1998; DaSilva and DosSantos, 2012). Lamina II: PKC $\gamma$  interneurons, at the border between noxious and innocuous tactile primary afferents, might thus regulate the cross-talk between sensory modalities. Receiving myelinated tactile primary afferent inputs, including A $\beta$ , at the lamina II/III border, they would act as gateway of local excitatory circuits that mediate mechanical allodynia.

## **CONFLICT OF INTEREST STATEMENT**

None of the authors has any conflict of interest to declare.

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## **ROLE OF AUTHORS**

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: CP, ML and RD. Acquisition of data: CP, RB and SP. Analysis and interpretation of data: AA, CP, ML and RD. Drafting of the manuscript: AA, CP, and RD. Obtained funding: ML and RD. Study supervision: RD.

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## FIGURE LEGENDS

**Figure 1:** Rat brain and spinal cord tissue preparation probed with anti-GABA<sub>A</sub> receptor  $\beta$ 2/ $\beta$ 3 (left), anti-Glycine receptor (middle), or without primary antibody (right). In the spinal cord, the two bands observed with the anti-GABA<sub>A</sub> receptor antibody correspond to the  $\beta$ 3 (57kDa), and  $\beta$ 2 (55kDa) subunits. Glycine receptors (48kDa) appear more abundant in the spinal cord than in the brain. No signal was detected in control experiments, after omitting the primary antibodies.

**Figure 2:** PKC $\gamma$  immunostaining in MDH. **A:** Fluorescence images from transverse sections through the MDH immunolabelled to reveal PKC $\gamma$  using antibody sc-211. Left and right hemisections are from the very same section which has been cut on the middle. The left hemisection was pre-incubated for 30 min with the peptide p-sc-211 (40 ng.ml<sup>-1</sup>) against which the antibody has been raised. Only the right hemisection displays the characteristic lamina II<sub>i</sub> PKC $\gamma$  immunostaining. Scale bar = 500  $\mu$ m. **B-D:** Fluorescent images from transverse sections through the MDH lamina II<sub>i</sub> immunolabelled to reveal PKC $\gamma$  using the polyclonal antibody sc-211 (B) and monoclonal antibody P8083 (C). As seen on the merged image (D), PKC $\gamma$  immunoreactive cells are immunolabelled by the two antibodies. Scale bar = 20  $\mu$ m.

**Figure 3.** Schematic representation of the 300 x 400  $\mu$ m cell counting window (pointed by arrow). Its median axe is positioned perpendicular to the MDH border (longest dotted line) and its dorsolateral end at the border between the white and grey matter. The center of this window (black dot) is set on the diagonal of a rectangle enclosing the whole MDH (black square). Scale bar = 500  $\mu$ m.

**Figure 4:** Rostro-caudal distribution of PKC $\gamma$  immunoreactivity within trigeminal MDH. **A-E:** PKC $\gamma$  immunoreactivity in transverse sections at various levels (800  $\mu\text{m}$  intervals) of the MDH, from its rostral (B: 0  $\mu\text{m}$ ; obex aperture) to its caudal end (E: -2400  $\mu\text{m}$ ). A transverse section at +400  $\mu\text{m}$ , where the MDH and Sp5I overlap with each other, is displayed in addition (A). On the right are the corresponding schematic drawings of laminar organization. It is clear that PKC $\gamma$  immunoreactivity occupies mainly the inner part of lamina II (lamina II<sub>i</sub>). Thick and dense at the rostral end of MDH (-2400  $\mu\text{m}$ ), the lamina II<sub>i</sub> band of PKC $\gamma$  immunoreactivity becomes thinner (-1600 and -800  $\mu\text{m}$ ) and scattered (0  $\mu\text{m}$ ) when moving rostrally. It has disappeared at +400  $\mu\text{m}$  where the MDH and Sp5I are overlapping and the laminar organization is lost. Scale bars = 500  $\mu\text{m}$ . I, lamina I. II, lamina II. III, lamina III. IV, lamina IV. V, lamina V. MDH, medullary dorsal horn. Sp5I, spinal trigeminal nucleus interpolaris.

**Figure 5.** Laminar distribution of NeuN and PKC $\gamma$  immunoreactive cells within lamina I-IV of MDH. **A-C:** Confocal images showing NeuN (A), PKC $\gamma$  (B) and merged NeuN and PKC $\gamma$  (C) immunoreactivities in laminae I, II<sub>o</sub>, II<sub>i</sub> and III-IV. Border between layers are shown with dotted lines. Scale bar = 50  $\mu\text{m}$ . **D:** Proportion of PKC $\gamma$  immunoreactive cells in laminae I-IV. More than half of the total number of PKC $\gamma$  immunoreactive cells are found in lamina II<sub>i</sub>. **E:** Bar histogram of the relative number (mean + SEM) of NeuN/PKC $\gamma$  immunoreactive neurons to the total number of NeuN immunoreactive neurons within each laminae I-IV. In lamina II<sub>i</sub>, PKC $\gamma$  immunoreactive cells represent about 1/3 of total neurons. Data are shown as mean  $\pm$  SEM (n = 4).

**Figure 6.** Synaptic inputs to PKC $\gamma$  immunoreactive interneurons. **A, B:** Axodendritic asymmetric (A) and axosomatic symmetric (B) synapses on a PKC $\gamma$  immunoreactive cell (DAB

reaction). Arrows point to active zones. Scale bars = 500 nm. Pre, presynaptic. Post, postsynaptic. N, nucleus.

**Figure 7.** Primary afferent inputs to PKC $\gamma$  immunoreactive interneurons. **A-C:** VGLUT3-immunostaining in the MDH. Light microscopic image through a transverse section of the MDH immunostained to reveal VGLUT3 (C) and the corresponding NeuroLucida reconstruction (D). Dashed lines represent lamina I-II<sub>o</sub>, II<sub>o</sub>-II<sub>i</sub> and II<sub>i</sub>-III borders. Fibers coming from the Lissauer tract project ventrali through the most superficial layers and form a dense plexus within the inner part of the lamina II. Scale bars = 20  $\mu$ m. **C:** Bar histogram of the normalized VGLUT3 immunoreactivity area to the total area of each lamina (I-III) (mean + SEM; n = 5). **D:** Electron microscopic image of an asymmetric synapse between a primary afferent and a PKC $\gamma$  immunoreactive interneuron (gold particles) in a type II glomerulus. Arrows cross asymmetric synapses between the presynaptic terminal and postsynaptic dendrites (stars). Scale bar = 500 nm. Pre, presynaptic. Post, postsynaptic.

**Figure 8.** GABA<sub>A</sub>ergic inputs to PKC $\gamma$  immunoreactive interneurons. **A:** Colocalization of GABA<sub>A</sub> receptor immunoreactivity (gold particles) in a PKC $\gamma$  immunoreactive interneuron (DAB reaction). Inset: higher magnification of the box. Scale bar = 1  $\mu$ m. **B-D:** Bar histograms (mean + SEM) of the distribution of gold particles signaling GABA<sub>A</sub> receptor immunoreactivity to plasma membranes in PKC $\gamma$  immunoreactive somatic (B, C) and dendritic (D) profiles. Distances are between the midpoint of each gold particle and the centre line of the associated plasma membrane. Extra- and intracellular positions are displayed together. Measurements were grouped in 200 nm (B) and 50 nm wide bins (C, D). Histogram in C is the same as in B but for the first 400 nm from somatic membranes. **E:** Areal density (number of particles.  $\mu$ m<sup>-2</sup>) of gold particles signaling GABA<sub>A</sub> receptor immunoreactivity in

PKC $\gamma$  immunoreactive somatic (left) and dendritic (right) profiles. Data are shown as both individual values (filled squares) and mean  $\pm$  SEM (horizontal lines) (soma, n = 7; dendrites, n = 20).

**Figure 9.** Glycinergic inputs to PKC $\gamma$  immunoreactive interneurons. **A:** Colocalization of glycine receptor immunoreactivity (gold particles) in a PKC $\gamma$  immunoreactive interneuron (DAB reaction). Inset: higher magnification of the box. Scale bar = 2  $\mu$ m. **B-D:** Bar histograms (mean + SEM) of the distribution of gold particles signaling glycine receptor immunoreactivity to plasma membranes in PKC $\gamma$  immunoreactive somatic (B, C) and dendritic (D) profiles. Distances are between the midpoint of each gold particle and the centre line of the associated plasma membrane. Extra- and intracellular positions are displayed together. Measurements were grouped in 200 nm (B) and 50 nm wide bins (C, D). Histogram in C is the same as in B but for the first 400 nm from somatic membranes. **E:** Areal density (number of particles.  $\mu$ m<sup>-2</sup>) of gold particles signaling glycine receptor immunoreactivity in PKC $\gamma$  immunoreactive somatic (left) and dendritic (right) profiles. Data are shown as both individual values (filled squares) and mean  $\pm$  SEM (horizontal lines) (soma, n = 7; dendrites, n = 20).

**Figure 10.** Subcellular localization of PKC $\gamma$  immunoreactivity at the electron microscopic level. **A, B:** PKC $\gamma$  immunoreactivity (DAB reaction) in a fusiform (A) and egg-shaped (B) PKC $\gamma$  immunoreactive interneurons. Scale bars = 2  $\mu$ m. **C.** Gold particles signaling PKC $\gamma$  immunoreactivity in dendrites. Scale bars = 200 nm. Both techniques suggest that PKC $\gamma$  immunoreactivity is predominantly located to plasma membranes (white arrows in A, B). **D.** Bar histogram (mean + SEM) of the distribution of gold particles signaling PKC $\gamma$  immunoreactivity to plasma membranes. Distances are between the midpoint of each gold

particle and the centre line of the associated plasma membrane. Measurements were grouped in 10 nm wide bins. **E**: Example of clustered gold particles signaling PKC $\gamma$  immunoreactivity on plasma membranes close to, but not on, synaptic densities. **F**: Bar histogram (mean + SEM) of the distribution of gold particles signaling PKC $\gamma$  immunoreactivity to synaptic clefts. Distances are between the midpoint of each gold particle and the middle of the synaptic density. Measurements were grouped in 50 nm wide bins. PKC $\gamma$  immunoreactivity was very rarely encountered in active zones but frequently formed clusters in the perisynaptic space. Extra- and intracellular positions are displayed together. . Arrows in C and E show putative endosomes, frequently located close to asymmetric synapses. N, nucleus. C, centrosome.

**Figure 11.** Subcellular localization of PKC $\gamma$  immunoreactivity in MDH lamina II<sub>i</sub> interneurons and cerebellar Purkinje cells. **A, C**: Light microscopic image from a transverse section through MDH (A) and a coronal section through the cerebellar cortex (C) immunostained for PKC $\gamma$ . Insets show higher magnifications of the respective selected neurons. Arrows point to nuclei, double arrows to plasma membranes and arrowheads to the cytoplasm. Scale bars = 20  $\mu$ m. **B, D, F, G**: Confocal images of PKC $\gamma$  immunoreactivity (B, D) and PKC $\gamma$  gray levels (F, G) of a MDH lamina II<sub>i</sub> interneuron (B, F) and cerebellar Purkinje cell (D, G). Gray values in F and G have been measured along the black lines in B and C, respectively. Vertical lines in B, D indicate changes in cellular compartments: plasma membrane to cytoplasm (full lines), cytoplasm to presumably nucleus (dotted lines). Changes in cell compartments in F, G are indicated with dotted lines. Scale bars in B, D = 10  $\mu$ m. **E**: Bar histogram of the relative subcellular localization (plasma membrane: filled bars; perikarion: empty bars) of PKC $\gamma$  immunoreactivity in MDH lamina II<sub>i</sub> interneurons and Purkinje cells. Data are shown as mean  $\pm$  SEM (n = 10 per group). \*\*\*, p<0.0001. \*\*, p<0.001. Mb, plasma membrane. Cyt, Cytoplasm

**GABA<sub>A</sub>****GlyR****Non-immune serum**

Brain

Spinal cord

Brain

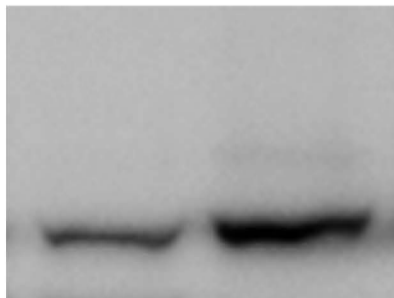
Spinal cord

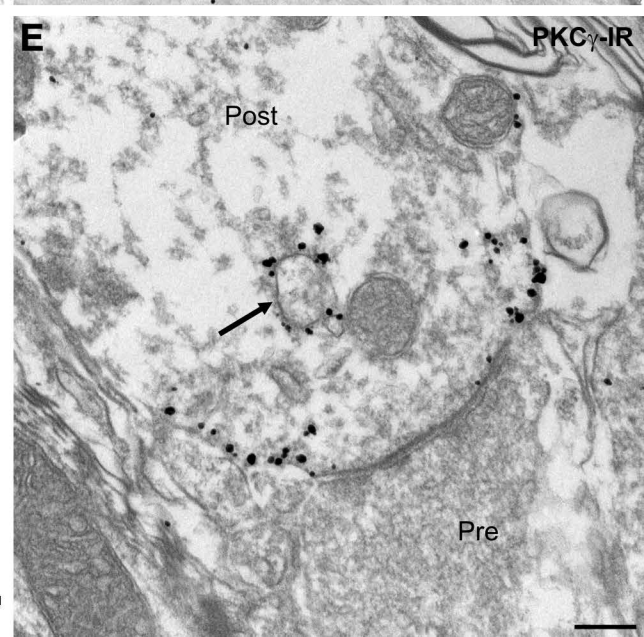
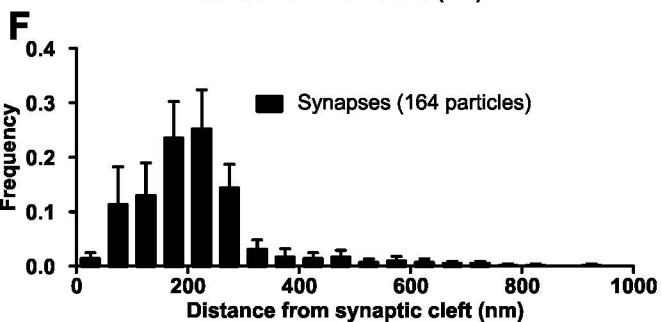
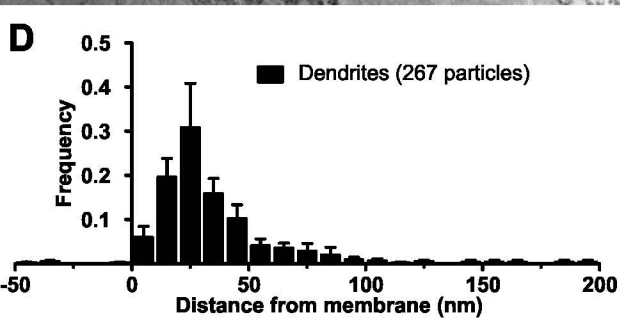
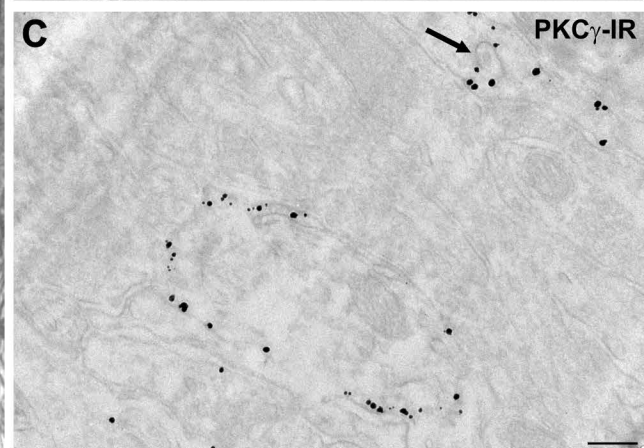
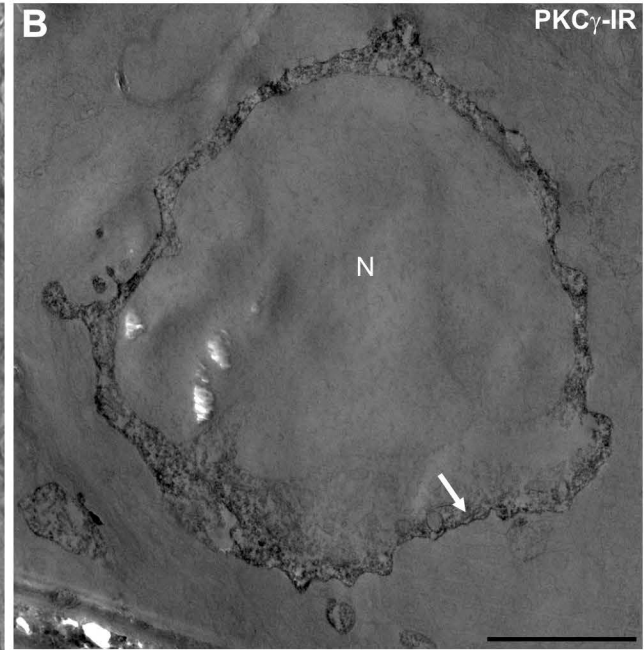
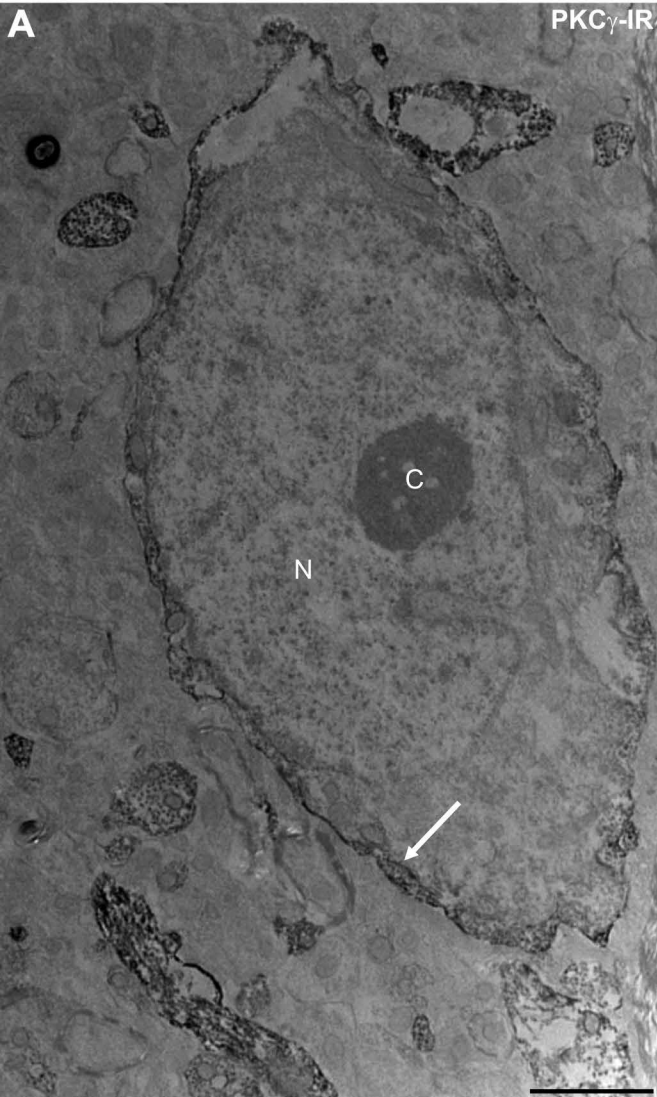
Brain

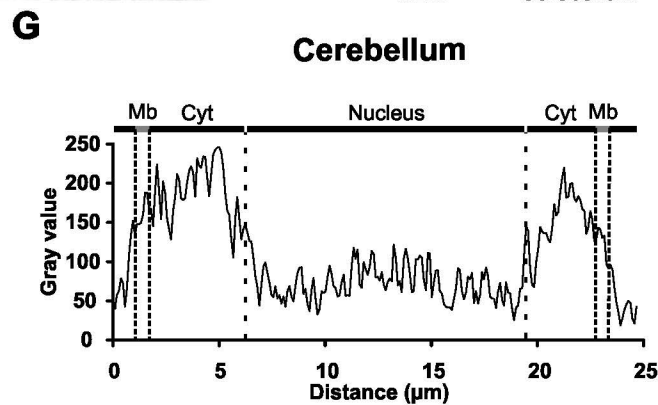
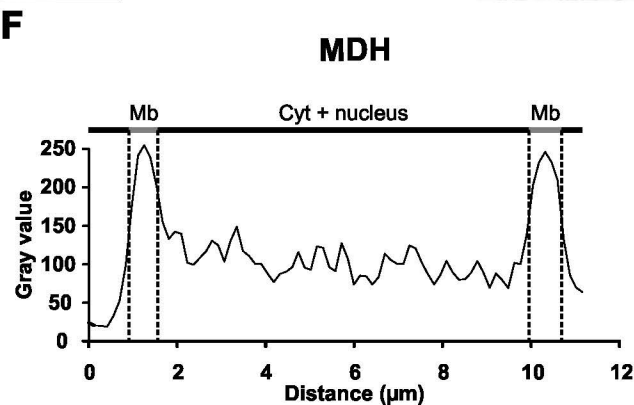
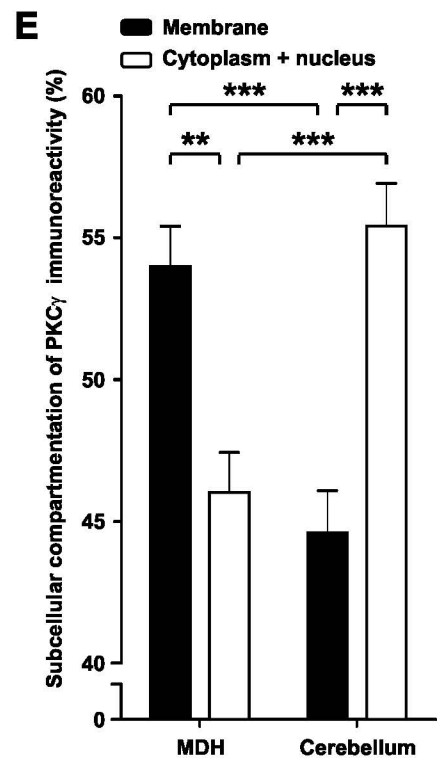
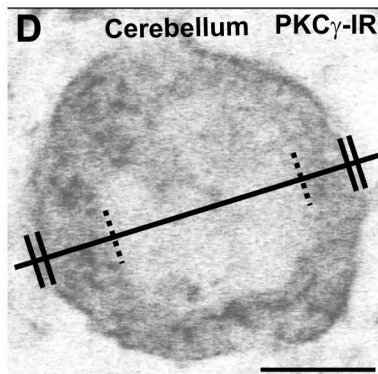
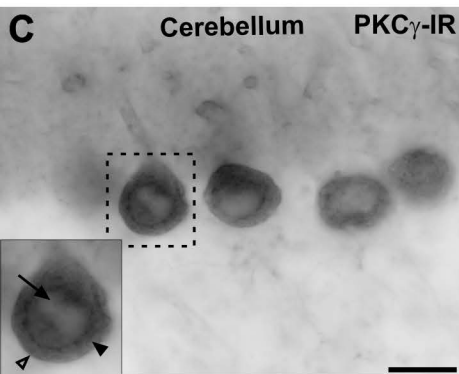
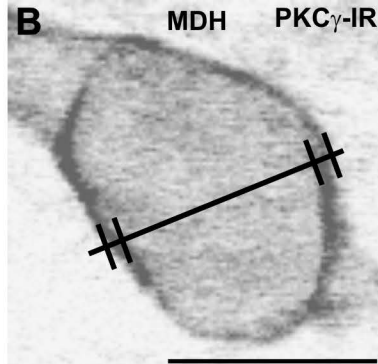
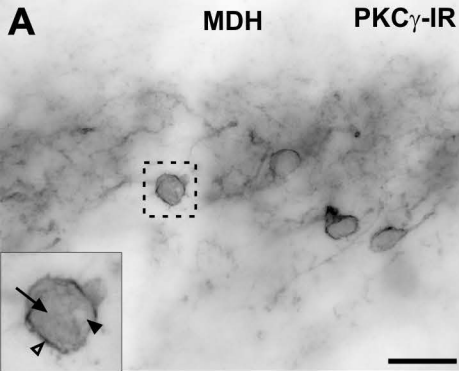
Spinal cord

57 kDa-

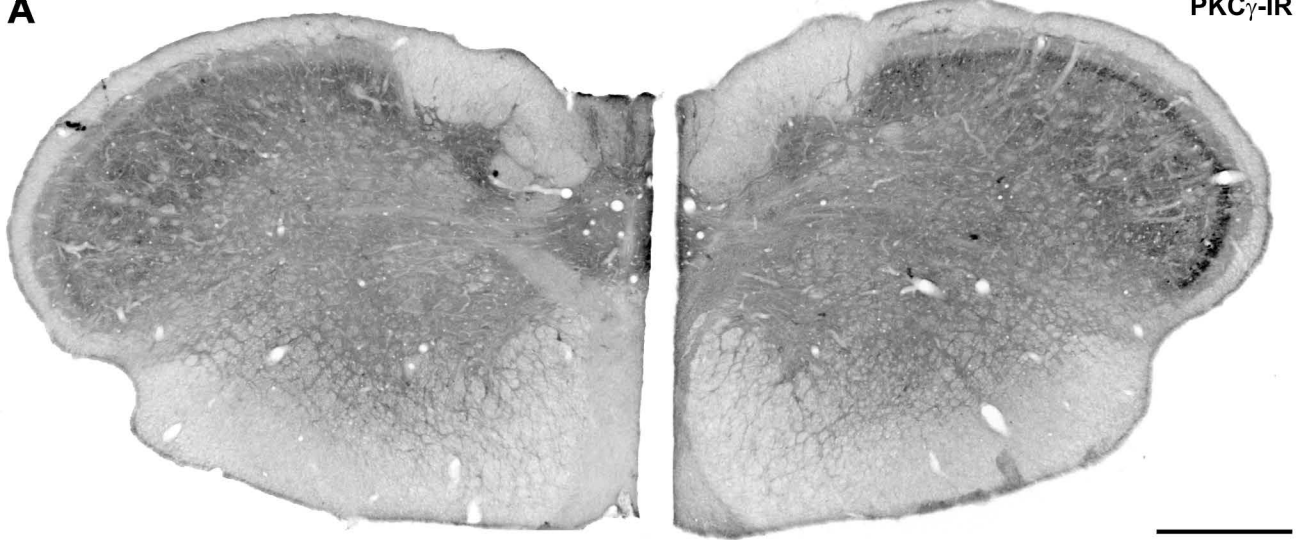
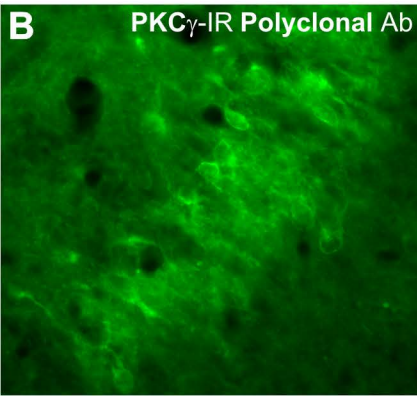
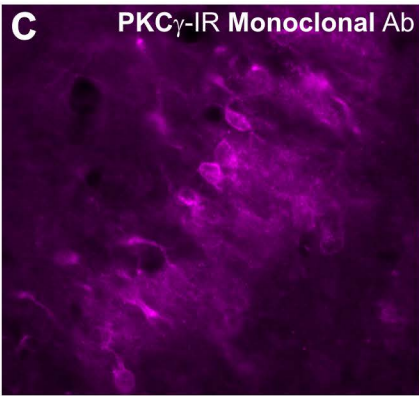
50 kDa-









**A****PKC $\gamma$ -IR****B****PKC $\gamma$ -IR Polyclonal Ab****C****PKC $\gamma$ -IR Monoclonal Ab****D****Merged**